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TITLE: MT 2A Phosphorylation by PKC Mu/PKD Influences Chemosensitivity to Cisplatin in Prostate Cancer

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14. ABSTRACT The metallothioneins (MT) are a family of small molecular weight trace metal and free radical scavenging proteins well established to play a role in resistance to chemotherapy and radiation in human cancer. MT gene expression is up regulated in response to the presence of heavy metal ions such as zinc. The activation of MT gene expression in response to zinc treatment in LNCaP and C4-2 prostate cancer (PC) cells was shown by western blotting and DNA microarray analysis. Chemotherapy and radiation sensitivity assays of cells following treatment with cisplatin or radiation were performed in the presence, or absence, of 150μM $ZnSO_4$ and cell viability measured after 72 hours by MTS viability, clonogenic and flow cytometry assays. Increasing concentrations of $ZnSO_4$ up regulated MT expression in a dose dependent manner. Microarray analysis demonstrated specific increase in MT expression. Cells treated with zinc demonstrated a significantly decreased sensitivity to cisplatin compared to controls ($p < 0.05$). We have established a physiological in vitro cell line model of MT induction using Zn, which is significantly associated with resistance to cisplatin chemotherapy in PC. Immunohistochemistry (IHC) analysis for MT expression in human prostate cancer specimens confirmed nuclear and cytoplasmic expression of MT in majority of specimens. However, there was no significant difference in expression between various grades of PC.

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THIRD ANNUAL REPORT

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Title: MT 2A Phosphorylation by PKCMu/PKD Influences Chemosensitivity to Cisplatin in Prostate Cancer.

Introduction

Because the PI has moved to University of Massachusetts, Worcester, MA from University of Nebraska, Omaha, NE in March of 2006, work on this project started in May 2007 following appropriate transfer of grant to University of Massachusetts. Herein, we report on the progress made from June-November, 2007.

Our preliminary studies had demonstrated that kinase domain of Protein kinase D1, a novel serine threonine kinase, interacts with Metallothionein 2A (MT 2A), which belongs to the family of small molecular weight proteins called metallothioneins (MT) that scavenge trace metals and free radicals and are associated with resistance to chemotherapy and radiation in human cancers [1]. Our studies further demonstrated the C4-2 prostate cancer cells that express higher levels of MT compared to its parental LNCaP cells [2]are selectively more resistant to trace metal containing chemotherapy agent cisplatin compared to LNCaP cells. Our *in vitro* experiments also demonstrated that MT 2A was phosphorylated by PKD1. Therefore we hypothesized ""*Alteration in MT 2A expression influences chemoresistance to cisplatin in prostate cancer. PKC Mu/PKD kinase activity influences sensitivity to cisplatin by MT 2A phosphorylation in prostate cancer. The expression of MT 2A is quantitatively increased in progressive human prostate cancer"*.

We proposed to establish the stated hypothesis through 3 aims.

Aim 1. To determine that alteration in MT 2A expression influences resistance to cisplatin in prostate cancer.

Aim 2. Inhibition of PKC Mu/PKD kinase activity and its influences on chemoresistance in prostate cancer cells by modulating the phosphorylation of MT 2A.

Aim 3. To quantify and qualitatively evaluate MT 2A protein expression in progressive human prostate cancer.

Body

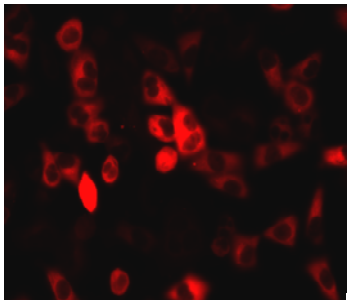
Task 1: *To determine that alteration in MT 2A expression influences resistance to cisplatin in prostate cancer.*

We reported completion of Aim 1 in our second annual report, which demonstrated that alteration in MT expression influences resistance to cisplatin in prostate cancer.

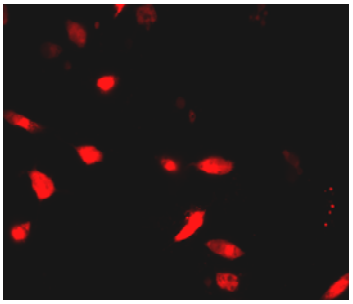
Task 2. Inhibition of PKC Mu/PKD kinase activity and its influences on chemoresistance in prostate cancer cells by modulating the phosphorylation of MT 2A.

a) Experiment 1: *Comparison of sensitivity to cisplatin of parental and MT 2A transfected LNCaP cells following treatment with PKC selective inhibitors by MTS assay*.

We created LNCaP cell lines that stably express wild type MT2A and nuclear localized MT2A (Fig.1).



LNCaP- pDsRed/MT-2A wt



LNCaP- pDsRed/MT-2A Ncl

Fig.1. LNCaP stable cell lines that express wild type (Left) and nuclear localized MT2A (Right). The MT2A is tagged by red fluorescence protein. Images were taken by an Olympus IX51 fluorescence microscope at 200X.

The stable cells were selected by Fluorescence Activating Cell Ssorting (FACS) for pools of at least 10,000 cells, so the population of each cell line is a mixture of various genomic integrations of the MT2A expression vectors. We have used the cell lines for cisplatin sensitivity assays.

Actively growing LNCaP and MT 2A transfected LNCaP cells were plated at 5000 cells per well of a 96 well plate in FBS free RPMI for 24 hours, and media was then changed to RMPI media with 10% FBS and treated cells by adding cisplatin with various concentrations of 0, 1, 5, 10, 20 and 50 mM. Growth and viability assessed by MTS assay at 3 days. Each experiment was carried out in triplicate (Fig 2).

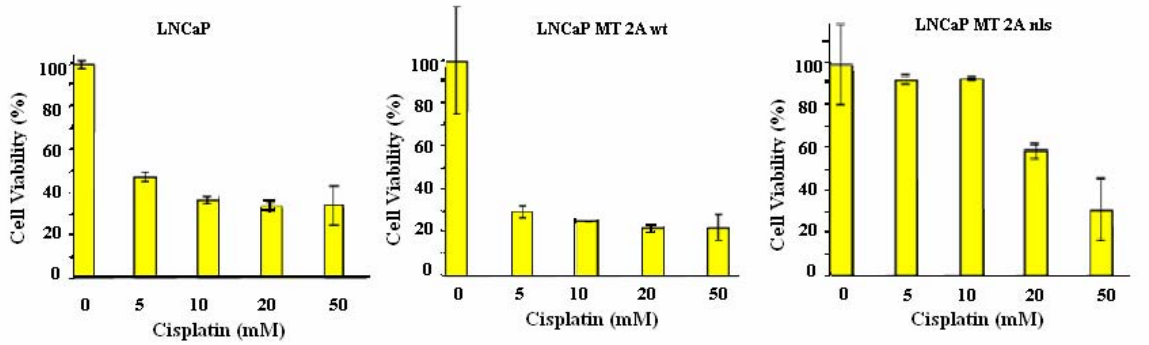


Fig.2. LNCaP cells with Nuclear MT2A, but not increased cytoplasmic expression of MT2A, demonstrate increased resistance to cisplatin treatment

As seen in Fig.2, parental LnCaP cells are sensitive to 5 uM of Cisplatin at which about 50% cells are dead (left panel). Wild type MT2A, which mainly remains in cytoplasm (Fig. 1) did not provide protection against cisplatin (middle panel). In contrast, LNCaP cells nuclear MT2A were not sensitive to 10 uM cisplatin (right panel). Even in the presence of up to 50 uM cisplatin, the survival rate was still higher compared to controls. These results demonstrate that only MT2A in nucleus can protect LNCaP cells from cisplatin.

PKD selective inhibitor Go6976 was tested for its influence on LNCaP cell viability and resistance to cisplatin. Parental and MT2A stable cell lines were treated with a serial concentration of cisplatin in the presence or absence of Go6976. MTS assay were carried out at 3 days. As seen in Fig. 3, in the absence of cisplatin, Go6976 (0.1 uM) shows cytotoxicity to parental (left panel) and wild type MT2A LNCaP (middle panel) cells, either by inhibiting of PKD or by general toxicity. However, LNCaP cells with nuclear MT2A are resistant to the

toxicity in the absence of cisplatin (right panel), further suggesting that nuclear MT2A play an important role in detoxification. In the presence of cisplatin, LNCaP cells with nuclear MT2A were sensitive to the even lowest concentration of cisplatin (5 uM), suggesting that inhibition of PKD may reduce cell viability (compare right panels in Figs.2 and 3).

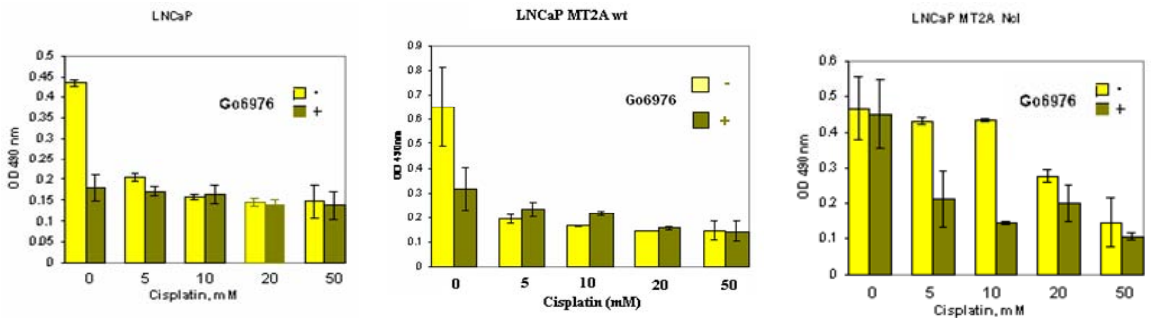


Fig.3. Nuclear expression of MT2A prevents decreased cell viability caused by PKD1 inhibitor GO6976 in LNCaP cells.

Experiment 2: *Determination of MT 2A phosphorylation using phosphoserine specific antibodies and kinase assays in cells treated with PKC inhibitors*;

We attempted to demonstrate MT2A phosphorylation by PKD1 using GST tagged MT2A. As demonstrated below in Fig 4, our study failed to demonstrate MT2A by PKD1 suggesting that PKD1 may influence MT function through subcellular localization.

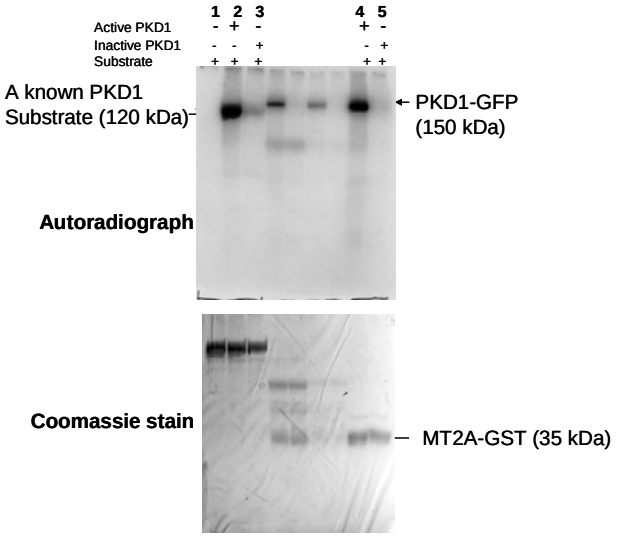


Fig.4. PKD1 does not phosphorylate MT2A in vitro. GST-tagged MT2A was purified from *E.coli*. Active and kinase-dead (inactive) PKD1 were purified from human 293T cells that were transfected with correspondent constructs. Lanes 1-3, active PKD1 was able to phosphorylate a known substrate, suggesting that our assay system was working. In lanes 4 and 5, active PKD1 failed to phosphorylate MT2A. Top panel is an autoradiograph and lower panel is a gel stain picture.

Task 3: *To quantify and qualitatively evaluate MT 2A protein expression in progressive human prostate cancer.*

We proposed to carry out study of expression of MT expression in human parafinized prostate tissue using immunohistochemistry and Quantitative Florescence Image Analysis (QFIA). Using 31 prostate cancer tissue samples, we reported in our first annual report that there was no significant correlation between MT expression and Gleason grade, disease stage or serum PSA.

Furthermore, we reported optimization of precise MT measurements in LNCaP cells by QFIA. Since we have made multiples attempts to standardize conditions to measure MT expression in prostate tissue and have been unsuccessful. It is likely that we will be unable to quantify MT expression precisely in prostate tissue as originally proposed.

Key Research Accomplishments

MT2A contributes to resistance to cisplatin in prostate cancer cells through nuclear localization. MT2A phosphorylation by PKD1 is not demonstrable, suggesting that PKD1 may contribute to cisplatin resistance by altering subcellular localization of MT.

Reportable Outcomes

1. David J Smith, Meena Jaggi, Wenguang Zhang, Anton Galich, Cheng Du, Samuel Sterrett, Lynette Smith and **K.C. Balaji**; Metallothioneins and Resistance to Cisplatin and Radiation in Prostate Cancer. ***Urology***, Vol 67, 6, 1341-47, 2006.

2. Narayini Narassa and **K.C. Balaji**; Metallothioneins and Prostate Cancer; "Metallothioneins in Biochemistry and Pathology" by Paolo Zatta: 2008, World Scientific Publishing Company, Singapore; Preorder on Amazon.com; http://www.amazon.com/METALLOTHIONEINS-BIOCHEMISTRY-PATHOLOGY-Paolo-Zatta/dp/9812778934/ref=sr_1_3?ie=UTF8&s=books&qid=1202161096&sr=1-3 ; (**In Press**, 2008)

Conclusions

Nuclear MT expression is associated with resistance to cisplatin chemotherapy in human prostate cancer cells.

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1. Ebadi, M. & Iversen, P. L. Metallothionein in carcinogenesis and cancer chemotherapy. *Gen Pharmacol* 25, 1297-310. (1994).

2. Rao, P. S., Jaggi, M., Smith, D. J., Hemstreet, G. P. & Balaji, K. C. Metallothionein 2A interacts with the kinase domain of PKCmu in prostate cancer. *Biochem Biophys Res Commun* 310, 1032-8 (2003).